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Transgenic rabbit producing human growth hormone in milk

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Abstract. The gene construct *WAP(6xHisThr):hGH* containing the entire human growth hormone gene (*hGH*) under the rat whey acidic protein (*WAP*) promoter regulating the expression in mammary glands of mammals was prepared. The 5' end of the gene was modified by the addition of a sequence encoding six histidine residues and a sequence recognized by thrombin. The gene construct was introduced by microinjection into the male pronucleus of a fertilized oocyte. The founder male rabbit was obtained with the transgene mapping to chromosome 7. The presence of the growth hormone was confirmed in samples of milk collected during the lactation of F1 generation females. The growth hormone can be easily purified by affinity chromatography and cleavage by thrombin to an active form.

Key words: acidic protein promoter, human growth hormone (hGH), microinjection, milk, rat whey, transgenic rabbit.

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Introduction

Human growth hormone (hGH) is required for normal growth in vertebrates; it participates in metabolism of mammals by stimulating protein synthesis and lipid degradation. Human growth hormone is composed of a single polypeptide chain of 191 amino acids with two internal disulfide bonds and a tertiary structure that includes four alpha helices arranged in an antiparallel fashion. The genomic sequence of *hGH* encompasses 1632 nucleotides including 5 exons.

In 1982 a gene construct containing the mouse metallothionein promoter (*mMT*) and the rat growth hormone gene (*rGH*) was introduced by microinjection to mouse zygotes (PALMITER et al. 1982). Females generated in that experiment were infertile, but males were able to transfer a foreign gene to the offspring, which was characterized by an increased growth rate due to the constitutive overexpression of rat growth hormone. Growth hormone concentration in transgenic mice was 500-fold higher and their weight doubled in comparison with control mice. Every cell contained 30 copies of the rat growth hormone gene. Interesting results were obtained by Australian scientists who introduced to the pig genome additional copies of the pig growth hormone gene (*pGH*) under control of the metallothionein gene promoter (*hMT*). Transgenic animals generated by this group had a significantly increased growth rate resulting from the overexpression of the growth hormone gene (*pGH*). The arrangements on the introduction of these animals into commercial market are being conducted (NOTTLE et al. 1999).

The only hormone preparation applied in dwarfism treatment and recognized by the World Health Organization (WHO), is peptide isolated from bacteria. The hormone preparation has excellent biological characteristics, but is relatively expensive (PHILLIPS 1995). Growth hormone can be easily obtained by extraction from human pituitary glands. The cost of hormone isolated in that way is not high, but the availability of material is limited and there is a risk of transfer of prions or other infectious agents. An alternative way is a possibility to use transgenic animals as bioreactors producing the human growth hormone in milk. A construct containing regulatory elements of the rabbit *WAP* gene linked with the human growth hormone gene (6.3 kb) was applied to generate transgenic mice (DEVINOY et al. 1994). The level of hormone reached the concentration of 4-22 mg/ml. An equally efficient expression of growth hormone in the milk of transgenic mice was achieved under control of the rat β-casein gene promoter (LEE et al. 1996). Transgenic mice with the construct containing the 3' end sequence of the *hGH* gene produced growth hormone more efficiently than mice with the construct containing the 3'end sequence of the rat β-casein gene. The 3'end of the *hGH* gene was found to be very important for efficient expression.

Transgenic rabbits containing a fusion gene (the mouse whey acidic protein gene promoter linked to the human growth hormone) were obtained by LIMONTA et al. (1995). They transfected 552 single cell embryos and achieved 72% survi-

vors. The embryos were transferred to wombs of 31 synchronized females and 51 rabbits were born. Forty-seven rabbits were monitored for the presence of the transgene and eleven were found to be transgenic. The efficiency of transgenesis was 23.4% (in relation to the litter number) and 2% (as for the number of microinjected eggs). The growth hormone concentration in milk reached 50 mg/ml in the best case.

The aim of the present studies was to prepare a human growth hormone gene construct and to generate a transgenic rabbit producing the human growth hormone in the mammary gland. The 5' end of the human growth hormone gene was modified by adding the sequence coding six histidine residues and the sequence recognized by thrombin in aim to simplify the purification of hormone. This modification enables the purification of peptide from milk by the application of affinity chromatography based on the affinity of histidine to nickel ions and the cleavage by thrombin results in a biologically active form of the growth hormone.

Material and methods

Cloning

PCR primers (TibMolBiol) were designed based on the human growth hormone gene sequence available in GeneBank (Accession M13438) at NCBI. Human genomic DNA isolated from peripheral blood lymphocytes was used as a template for PCR. The PCR mixture contained 10 pmoles of each primer; 7.5 nmoles of dNTP and 0.5 U of Taq polymerase in a final volume of 50 µl. PCR was performed as follows: denaturation – 92°C, 60 s; annealing – 54°C, 60 s; synthesis – 72°C, 3 min., in 35 cycles. PCR products were analyzed in 1.5% agarose gel and then digested with restriction enzymes (*Nco*I and *Hind*III) at 37°C for 1 hour. The digested products were then purified on Wizard columns (Promega) and ligated with the vector containing the rat *WAP* promoter within *Nco*I and *Hind*III restriction sites. Transformation was carried out in the *E.coli* DH5α strain. The nucleotide sequence of the gene construct was determined using a cycling sequencing kit and ALFExpress sequencer (Pharmacia Biotech).

Microinjection

The experiments were performed on New Zealand rabbits. Exogenous DNA in the form of the *WAP(6xHisThr):hGH* gene construct was introduced to the male pronucleus of a fertilized rabbit egg. Microinjection was performed using two pairs of micromanipulators (one for zygotes manipulation, the other for injection) and reverse microscopy. The volume of the introduced DNA was usually 2-5 pl, injected till a moment in which the pronucleus increased its volume by approximately 50%. The number of introduced DNA copies varied from several to a few

dozen thousand, depending on the volume of the microinjected DNA solution, which was at the concentration of 6 ng/µl. Following injection zygotes were segregated, the best zygotes were selected and transferred to the oviducts of synchronized recipients.

Screening for the presence of transgene

The screening procedure involved the isolation of genomic DNA from the ear microsections of rabbits and the amplification of two PCR-fragments encompassing the promoter-gene junction. One of each pairs of primers was labeled at the 5' end with the fluorescent marker Cy5. Forward primers were located in the *WAP* promoter region and reverse primers in the region coding the growth hormone. PCR product of 313 bp was amplified with WHGH2F (5'–Cy5–AGTCTTCCT-CCTGTGGGTC–3') and WHGH2R (5'–TCTCTCTCCATCCCTCCAG–3') primers, whereas the 524 bp fragment was obtained with WHGH1F (5'–Cy5–GTCCCAACCCAACCATTC–3') and WHGH1R (5'–TGGCGATAC-TCACATTCAGA–3') primers.

Following isolation of the DNA with Proteinase K, two pairs of PCR primers were used in two reactions. The reaction contained: 100 ng DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 7.5 pmol of each primer and 0.7 U Taq polymerase in a final volume of 20 µl. PCR was performed using the following conditions: denaturation – 94°C, 45 s; annealing – 55°C, 45 s; synthesis – 72°C, 90 s; 30 cycles.

PCR products were fractionated in 6% polyacrylamide gel (19:1, AA:BA) under denaturing conditions on the ALFExpress sequencer. Two microliter aliquots of PCR products were combined with the loading buffer and internal markers (113 bp and 268 bp). For the analysis of results Fragment Manager software (Pharmacia Biotech) was applied.

Karyotype analysis and cytogenetic methods for transgene mapping

Karyotype analysis of rabbits was performed by the routine method of the GTG-banding pattern. The major method applied for the mapping of the transgene was FISH (fluorescence in situ hybridization), in which the transgene DNA probe [*WAP(6xHisThr):hGH*] was labeled with biotin (Bio-dUTP) by nick translation (Boehringer Mannheim). For the detection of signal on chromosomes, following hybridization, samples were incubated in several steps with avidin labeled with fluoresceine (avidin-FITC), anti-avidin antibodies and avidin-FITC. For the observation of the transgene signal under a microscope samples were stained with DABCO/PI or DABCO/DAPI.

Detection of growth hormone in milk

The detection of the growth hormone in milk was performed using the immunoradiometric kit hGH-IRMA from Polatom. Total unfractionated milk

samples from every individual female of the F1 generation were collected during the lactation period and tested separately, or a test was performed on a pool of milk collected from a single female during the whole lactation period. The tests were carried out mostly in total milk samples and also in casein and whey fractions. As a control serum was tested for the presence of hGH.

Results

The *hGH* gene construct (1563 bp) was amplified by PCR with the forward primer containing the *Nco*I restriction site, the sequence coding 6 histidine residues, the thrombin recognition site and 20 nucleotides complementary to the 5' end of the *hGH* gene and the reverse primer complementary to the 3' end of the *hGH* gene and containing the *Hind*III restriction site. *WAP(6xHisThr):hGH* plasmid DNA was digested with *Hind*III and purified. The construct prepared in this way (at the concentration of 6 ng/μl) was introduced to 291 rabbit zygotes by the standard method of microinjection to male pronuclei of fertilized eggs. Two hundred sixty two zygotes were chosen for transfer to 11 synchronized recipients and 22 alive rabbits were born.

The screening procedure involving 313 bp and 524 bp PCR products was applied to identify transgenic rabbits. One individual male (number 61) from the F0

Figure 1. Transgenic rabbit with the human growth hormone gene. Two month old New Zealand male rabbit number 61 of the F0 generation, a founder of the transgenic line. The rabbit was generated following the implantation of a zygote microinjected with the *WAP(6xHisThr):hGH* gene construct. The founder and his offspring showed no changes in phenotype and behavior.

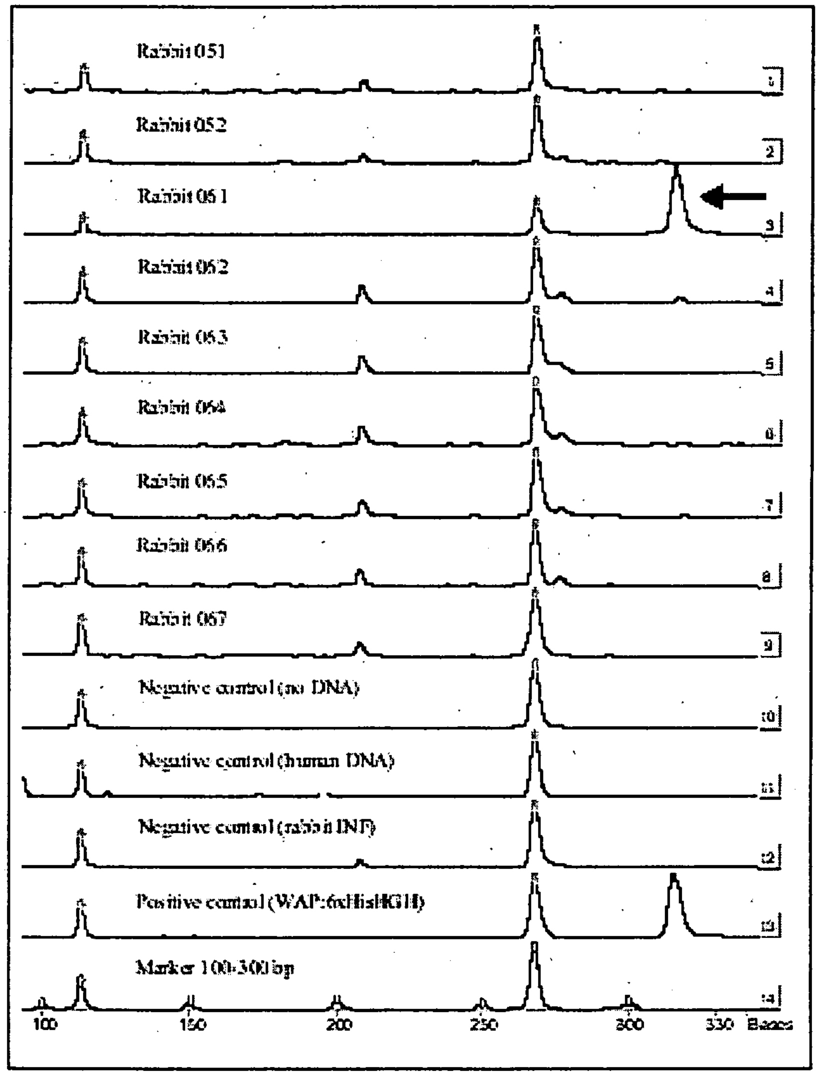

Figure 2. Screening for hGH transgene in population of F0 rabbits. Screening for the transgene was performed by PCR encompassing 313 bp or 524 bp (data not shown) DNA fragments. PCR primers were located upstream and downstream of the histidine tag and thrombine recognition site of the gene construct. An arrow indicates the presence of the transgene in rabbit number 61 of the same size as input DNA fragment (positive control in line 13). PCR observation was confirmed by sequencing.

generation (Figure 1) was found to have the transgene incorporated. The screening results of fragment 313 bp are presented in Figure 2, similar data for 524 bp product are not shown. The founder and his offspring showed no changes in phenotype and behavior. When male 61 reached sexual maturity, his sperm was collected and used for artificial insemination of non-transgenic females. As a result of this procedure 40 rabbits of the F1 generation were generated. The presence of the transgene was confirmed by PCR screening in 12 individuals (6 males and 6 females).

Figure 3. Mapping of the human growth hormone gene in a transgenic female rabbit of the F1 generation (No. 08). The location of *WAP(6xHisThr):hGH* specific sequences (green color signal) on the q26-27 region of chromosome 7 is demonstrated by FISH.

Transgenic animals were subjected to classical and molecular cytogenetic analysis after primary cell lines from skin fibroblasts of F1 transgenic heterozygous animals were established. Based on the GTG-banding pattern no chromosomal aberrations in rabbits were observed. FISH with DAPI staining (visualization of the G-banding pattern) enabled the mapping of transgene specific DNA sequences to the q26-27 region of chromosome 7. In all 12 rabbits confirmed to contain the transgene by PCR screening and subjected to FISH analysis, the location of the transgene was detected in the same q26-27 region of chromosome 7. In Figure 3 FISH mapping of the transgene in female No. 08 of the F1 generation (daughter of male founder No. 61) is presented.

When transgenic females of the F1 generation reached sexual maturity, they were inseminated with the semen of the transgenic male to obtain homozygotes.

Milk samples collected from F1 females during lactation were confirmed to be positive for the presence of hGH reaching the level of 0.5-1 micrograms per milliliter with 97% present in fat free milk (casein fraction – 53%; whey fraction – 44%). No hGH was observed in the serum of rabbits.

Discussion

Purification of proteins from blood or as in the case of the human growth hormone from pituitary glands is accompanied by the risk of contamination by human infectious agents. Concerning the spread of infectious agents such as HIV, HCV or the Creutzfeldt-Jacob disease it is of great importance to search for alternative biological systems, which enable the generation of proteins using a methodology avoiding these threats. Dutch researchers reported recently on the case of a man who developed the Creutzfeldt-Jacob disease 38 years after receiving the human derived growth hormone (CROES et al. 2002).

Proteins obtained directly from donors are usually available in very small amounts, which makes their purification more difficult and expensive. The application of such proteins as bio-pharmaceuticals is limited or even impossible due to their high cost. The introduction and application of alternative biological systems enable obtaining large amounts of proteins at a relatively low cost. Recombinant proteins can be generated in bacteria, yeast, fungi, transgenic plants, animal cell cultures and transgenic animals. Gigantism, diabetes and other disease symptoms were observed in transgenic mice, pigs and sheep expressing the human growth hormone in blood (PURSEL et al. 1989). However, blood can be used as a valuable source of certain proteins (MASSOUD et al. 1991, MIKKELSEN et al. 1992, SWANSON et al. 1992, DAVIES et al. 1993). The choice of the biological system for protein production depends mostly on the type of post-translational modifications – acetylation, hydroxylation, glycosylation, phosphorylation, γ-carboxylation, amidation and sometimes lipid residues attachement.

The growth hormone produced in bacteria is commonly used for the treatment of dwarfism. Although it is entirely a functional product it is different from the natural one, since proteins in prokaryotic cells are not subjected to glycosylation. Apparently, only animal cell cultures and also blood cells and mammary gland cells of transgenic animals can carry out all post-translational modifications precisely enough to form functional protein as it normally occurs in the human organism. Despite many advantages, the application of cell cultures is limited because of their relatively high cost and difficulties in culturing huge numbers of cells. At present milk seems to be the best source of recombinant proteins. The vectors directing the transgene expression in a particular tissue or all tissues must contain adequate regulatory elements. In case of a gene construct to be expressed in the mammary glands of transgenic animals the regulatory sequences of genes coding milk proteins are used. The expression of such genes is limited

to the mammary glands of animals and only during lactation. The milk protein genes, which promoter sequences are used most frequently, include: casein (α_{S1}, α_{S2}, β and κ), β-lactoglobulin, α-lactoalbumin and the whey acidic protein (*WAP*) gene. Casein occurs in most mammals in different concentrations, β-lactoglobulin is present mainly in the milk of ruminates, α-lactoalbumin is present in most mammals and whey acidic protein occurs especially in the milk of rodents. In our experiments the human growth hormone was not observed in the serum samples of all the transgenic animals, but was detected in unfractionated milk samples and in casein and whey fractions from the transgenic heterozygote females at the level of micrograms per milliliter.

There are still many problems concerning regulatory elements required for a high expression level of genes encoding milk proteins. The expression level of the transgene is unpredictable, however a higher expression level is observed more frequently in cases when genetic constructs contain the genomic sequence of a transgenic protein in comparison to constructs that are based on cDNA. The expression of the growth hormone in transgenic mice was studied extensively by Kopchick, who was able to answer a number of questions arising from transgenesis including lowered carcinogenesis in transgenic mammary glands (KOPCHICK et al. 1999, POLLAK et al. 2001). Our results open new intriguing areas of inquiry since new, completely characterized both on the molecular and cytogenetic levels, transgenic rabbits were obtained.

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